

The University of Chicago

THE DIFFERENTIATION OF CHICK LIMB
BUDS IN CHORIO-ALLANTOIC GRAFTS,
WITH SPECIAL REFERENCE
TO THE MUSCLES

A DISSERTATION SUBMITTED TO THE
GRADUATE FACULTY IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOÖLOGY

By
ELEANOR A. HUNT

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ELEANOR A. HUNT

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TWO TEXT FIGURES AND TWO PLATES (NINE FIGURES)

INTRODUCTION

This study is an attempt to analyze the differentiation of the limb bud of the chick embryo in chorio-allantoic grafts. Primarily it deals with the extent to which the chief components of the limb, bone and muscle, are self-differentiating systems. Particular attention was given to the muscle tissue, since very few observations have been reported on the development of muscle under experimental conditions either in previous work on the chick or in the extensive experiments with amphibian limbs. To a lesser extent it treats of, *a*) the contribution made by the somites to the musculature of the limb, and, *b*) the rôle of the nerve cord in the differentiation of the limbs. Although the results reported here are in many respects somewhat preliminary in nature, they indicate that possibly differentiation of muscle, but particularly the maintenance of normal muscle, is dependent upon innervation and perhaps also upon mechanical factors such as attachment and tension.

The writer is indebted to Prof. B. H. Willier for having suggested this problem and for his stimulating guidance and assistance throughout the course of the work.

¹ This work was carried on at the Hull Biological Laboratory at The University of Chicago under the direction of Dr. B. H. Willier.

MATERIAL AND METHODS

The method of growing tissue by transplanting it to the chorio-allantoic membrane of developing chicks has already been described fully by Willier ('24). In the present investigation hosts were incubated from eight to ten days and the age of the donors varied from forty-eight hours to seven days. The age most frequently used was about seventy-two hours. The tissue was allowed to remain on the host membrane from three to ten days, usually nine days. About 360 grafts were obtained, of which ninety were sectioned. These formed a series in which the tissues had a total time of growth of from six to sixteen days.

The eggs of White Leghorns were used almost entirely for host embryos and Black Minorcas and White Leghorns for donors. The possible advantages of combining tissues of differently pigmented embryos was considered, but was not made use of in these experiments. No difference in the development of the two kinds of eggs was noted and they will not be treated separately. Controls consisted of serial sections made of the limb regions of normal chick embryos of from two to nine days of incubation.

For the transplants both anterior and posterior limb buds of either side were used, but the majority were leg buds. To determine the degree of development of the donor at the time of grafting, the somites were counted in the younger embryos. In stages in which a definite limb bud had formed, notations were made only when the rudiment varied noticeably from the normal of that age.

Three types of grafts were employed. First, the rudiment of the limb by itself, i.e., somatopleure lateral to the somites extending from approximately somites 14 to 21 (level of wing) or somites 26 to 32 (level of leg) (fig. 1, *AB*). The exact anterior and posterior limits varied somewhat with successive grafts. Donors of two, three, and four days of incubation were used in this type of transplantation.

Secondly, the limb anlage was combined with one or more of the following parts—somites, nephrotome, neural tube

with notochord, and splanchnopleure (fig. 1, *CB*, *DE*). Implants were also made of the limb rudiment to which had been added portions of the neural tube from a different level. Donors for this type of implant were two and three days of age.

Thirdly, partial limb buds. This type of isolation applied chiefly to implants from older donors (three to seven days) in which the entire rudiment was frequently too large to be transplanted successfully. Cuts were made which divided

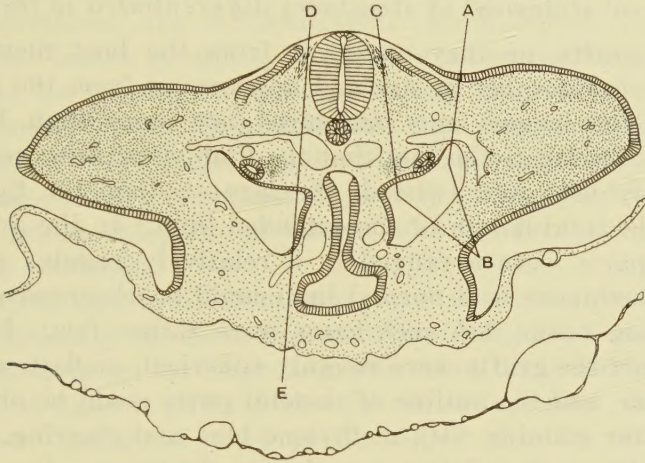


Fig. 1 Semidiagrammatic drawing of a cross-section through the leg buds of a seventy-two-hour chick embryo. The lines *AB*, *CB*, and *DE* indicate the chief cuts used for isolating the tissue for transplantation.

the limb bud into anterior and posterior parts, proximal and distal, and dorsal and ventral. Usually these parts were approximate halves.

At the end of the prescribed time most of the grafts were fixed in Carnoy's mixture (6-3-1), although Bouin's picroformol, Helly's modification of Zenker's fluid, and Benda's osmic-acid mixture were also used. The grafts were then stained in toto with methylene blue (National aniline chromatine blue violet or methylene blue N in 70 per cent alcohol) and decolorized with acid alcohol according to the method

used by Murray in studying limb grafts. Wintergreen oil was used for clearing, and many of the grafts were stored in this oil until a detailed study could be made. Before the tissue was embedded for sectioning, each graft was drawn to scale for purposes of orientation. The majority of the serial sections of grafts were stained with Heidenhain's iron hematoxylin, but Mallory's connective-tissue stain and methylene blue-eosin were also used.

EXPERIMENTAL DATA

1. General statement of structures differentiated in the grafts

The grafts, as they were cut from the host membrane, varied considerably in size and appearance from the normal limb. The largest ones measured not more than 15 mm. across. Owing to the fact that the limb structures were considerably bent and twisted, the largest diameter failed to equal the total length of the extended limb. In the grafts in which parts were arranged in a relatively regular manner the resemblance to a normal limb could be observed even in the living tissue, but such cases were rather few. For the most part the grafts were roughly spherical, or flattened and irregular, and the outline of skeletal parts could be observed only after staining with methylene blue and clearing. Vesicles, that are found in most chorio-allantoic grafts, tended here to distort the limbs, which in some cases were further obscured by the abundant development of feathers. In general, development was never entirely equal to that of the normal of a corresponding age, but some of the particularly successful grafts showed the degree of development that was possible under the experimental conditions.

The cartilaginous skeletal elements exhibited a great capacity for differentiation. In the best cases these cartilages represented essentially all of the skeletal elements of limb and girdle, to which in some specimens parts of vertebrae were attached. In other grafts various parts were lacking or could not be identified. In the least well-developed cases only two or three small, irregular cartilages were formed. Ossi-

fication generally occurred in quite a characteristic and normal manner, and in some of the older grafts, cartilage was almost entirely replaced by bone.

Microscopical examination showed that in most cases the muscle present in the grafts was subnormal with respect to the total amount developed, the grouping of fibers into bundles, and the differentiation of cross-striations in the fibers. In the control chicks definite fibers have appeared in the condensed mesenchymal premuscle mass by the sixth day. Dark-staining fibrillae then appear and during the seventh day cross-striations are differentiated. Even before this time there has been a grouping of fibers into distinct bundles. In most of the grafts, on the other hand, the earliest time of total incubation in which striations were observed was about ten days, if they formed at all. Fibrillae formed earlier than this and possibly striations were also present, but in many of the grafts the plane of section was not favorable for observing striations.

In some grafts distinct groups of fibers occurred which were evidently the forerunners of the definitive muscle bundles, but even in the most favorable cases, their identification would be difficult without careful reconstruction. Although distinctly delimited tendons developed distally about the digits, they usually could not be traced to any muscle group, any possible connection being lost in the intervening diffuse tissue. Muscles were not always attached directly to the skeletal parts, but might terminate in fibrous tissue or even in the loose mesenchyma or at the surface of the graft.

No accurate quantitative measurement was made of the total amount of muscle tissue, but in most cases it was quite obvious that the mass of normal muscle in the grafts was much less than in the control chicks. (The chief exceptions were several grafts having extensive innervation.) This was true even if some allowance was made for retardation in the graft and comparisons were made with controls which were a day or more younger than the total age of the grafted tissue.

In addition to the relatively normal muscle, a different sort of tissue was present which bore no resemblance to muscle, but was recognized as atypical muscle that had undergone degenerative changes. In many cases the presence of this tissue explained in part the small amounts of normal muscle observed. The appearance and occurrence of this atypical muscle will be discussed in subsequent pages.

The possible origin of the muscle tissue of the limb may well be mentioned here, since this method of experimentation might be expected to throw some light on this particular problem. Paterson ('88) determined from a morphological study that chick limb muscles form from the mesodermal cells of the somatopleure rather than from somitic derivatives, but Kaestner ('92) states that the ventral edges of the myotomes do contribute to the limb muscles. Lillie ('08, p. 188) states that "In later stages the myotomes are outgrowths into the limb buds and ventral body wall for the formation of the voluntary muscles of these parts." He implies, but does not definitely state, that this outgrowth occurs after the third day. In the amphibia it was first demonstrated by Byrnes ('98) that somites do not take part in the formation of limb musculature. Bardeen and Lewis ('01) found no trace of myotomes entering the limb bud in human embryos, but Lewis ('12, p. 480), in discussing the situation in general, states that the possibility of migration from the myotomes of cells of a mesenchymal character must be considered. It seems quite certain, however, that the epithelium of the myotome does not enter the limb bud. Butcher ('29) finds this is true in the rat. A similar condition probably obtains in the chick embryo, and only careful and critical experiments can establish the true origin of the limb musculature. Our grafts of limbs from three-day donors from which it was attempted to exclude somite tissue in the transplant contain muscle which does not appear to have developed to any less extent than in similar grafts in which somites were included. Although these results may seem to indicate that somites do not play a part in the formation of limb muscles, they may

not have been sufficiently controlled and cannot be considered as conclusive. Further experiments on early stages may prove enlightening. Murray ('28), for instance, found no muscles in grafts of rudiments from two-day embryos from which somite tissue (segmental plate) was excluded (six cases), although he had found it in previous experiments which did include somite tissue (one out of seven cases). He suggests (p. 282), therefore, that "perhaps the cells forming the muscles of the appendicular skeleton may originate from the somites. . . ."

Other structures that occurred in the grafts need not be described in detail. When nervous tissue, especially neural tube, was included in the implant, its relationship to the limb was essentially normal. Skin was frequently present, and not only a simple epidermis and dermis had developed, but also feathers and the characteristic smooth muscles of the skin which are attached to the feathers. Gut and nephric tissue differentiated very normally when their anlagen were included, as they frequently were. The presence or absence of all these structures except the nervous elements appeared not to have any effect on the limb development and therefore they will be disregarded in this report.

2. Muscle degeneration

Mention has already been made of the fact that the muscle developing in the grafts is not always normal in amount and in degree of differentiation. In addition to the relatively normal tissue, there are cells present in more than half of the grafts which are interpreted as degenerating muscle, the occurrence of which accounts at least partially for the scarcity of recognizable muscle tissue. In grafts preserved in the ordinary fixing fluids these atypical cells are filled with various-sized vacuoles (fig. 2, a and b). In the cases in which Benda's fluid was used, the cells were shown to contain droplets of fat which were blackened by the osmic acid (fig. 2, c and d). Subsequent treatment with hydrogen peroxide bleached the black from the osmicated tissue and gave a pic-

ture strikingly similar to that presented by tissue preserved by the usual methods which dissolve out the fat. Since no similar cells were detected in the normal control limbs, the changes occurring in the grafted tissue were considered evidence of a fatty degeneration peculiar to the grafts.

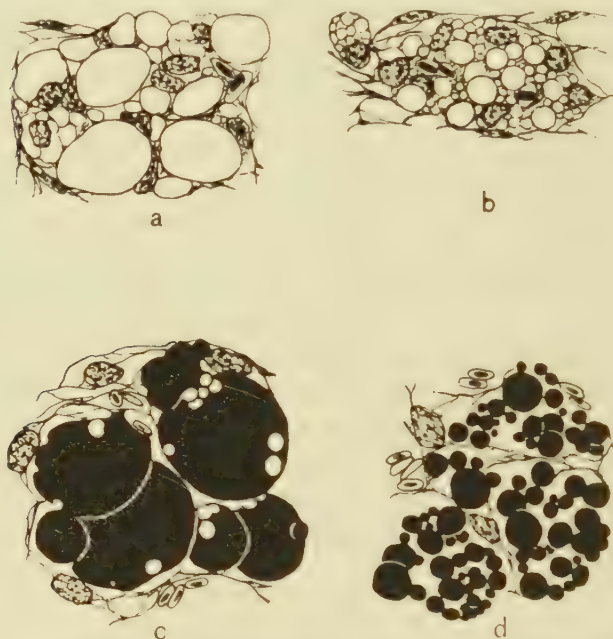


Fig. 2 Groups of degenerating muscle cells. a) Advanced stage with large vacuoles (representing fat droplets). b) Earlier stage with smaller vacuoles. c and d) Cells similar to those shown in a and b, but taken from a graft fixed in osmic acid. Camera-lucida drawings, $\times 600$.

There seems to be very little reason to doubt that this degenerating tissue actually represents muscle. The very fact that it is present in grafts where muscle is scarce or absent is in itself suggestive. Then the atypical cells are nearly always found in positions where muscle would be expected and they are frequently arranged in groups and masses which simulate muscle bundles. In grafts from the older donors the muscle is chiefly of the degenerating type,

and it is perhaps worth noting that when these rudiments are divided into basal and distal portions, the graft developing from the basal piece invariably has more degenerating tissue than the distal. This would be expected if the atypical cells in the former are considered to represent thigh musculature which is much more heavily developed than that of the shank. Vacuolated cells were not found around the digits where muscle does not occur normally.

One of the best proofs of the identity of the degenerating tissue is to be found in graft 23-18 (fig. 3). In this case the attachment of a mass of degenerating tissue to cartilage by means of a fibrous tendon is clearly shown. Usually the plane of section is not so favorable, but similar relationships have been observed in other grafts. Figure 4 might be mentioned as a case in which elongated groups of vacuolated cells occur in exactly the same relationship to the femur as a parallel bundle of muscle fibers and are attached directly to the cartilage instead of indirectly by a tendon.

The process of formation of the degenerating tissue cannot be completely outlined, as all stages have not been observed. Vacuolated cells are most prominent in the older grafts (i.e., after ten days' total growth) and occur without exception after twelve days. The only evidence of a transitional stage is that described in graft 11-16, which attained an age of ten days. On very close examination, some fibers show a vacuolated appearance as if the process were just starting. In another part of the graft is a mass of cells in which all fibrillar character is lost and the vacuoles are more prominent, although they evidently represent an early stage before the droplets have begun to coalesce. In other grafts some of the later stages may be followed without difficulty. At first the droplets are small (fig. 2, b and d), but as some of them flow together, they become irregular in size. When the vacuoles are very large and the cells are close together (fig. 2, a and c), they look like a mass of soap bubbles and cell boundaries can scarcely be detected. Nuclei do not show any very marked transformation.

The exact stage of development at which degeneration begins is of considerable interest. The appearance of the cells (ie., spherical rather than elongated and fibrillar) suggests that although muscle may have been determined, it had not undergone much differentiation before the degenerative process began, at least not to the stage of the formation of elongated, multinucleated fibers. However, the evidence presented by graft 11-16, which is considered to show an early stage of degeneration, indicates that the process may begin even at this relatively late period. Moreover, some of the transplants were from donors of six and seven days of age, so that muscle fibers must have undergone considerable differentiation before they were grafted. These also show degeneration. Graft 36-4 is from an implant of a mass of thigh muscle of a nine-day donor, and vacuolated cells are abundant, but no muscle fibers remain, although striated fibers must have been present when transplanted. In fully differentiated mammalian muscle pathologists recognize fatty degeneration by the accumulation of fat droplets among the fibrillae, finally filling up the entire space within the sarcolemma. There is no breaking up of multinucleated fibers into separate cells, however. Further investigation will be necessary to establish the details of the radical change which must occur in the grafted tissue.

3. Analysis of muscle differentiation in the grafts

In this section are recorded the general results for the several groups of grafts and also brief descriptions of certain specific cases which are considered representative. Since the condition of the muscle developed in the grafts appears to be somewhat related to innervation, they are grouped according to whether or not they contain nerve tissue.

A. Nerve tissue present. 1. Innervation of limb probable. These grafts include the cases in which some part of the neural cord was included in the transplant so that nerves have grown out and appear to be definitely related to limb parts. There are twenty-one grafts, of which four attained an age

of only about seven days and the limbs show such early differentiation that they are not included in the discussion. In these four grafts, however, there are no signs of degeneration in the mesenchyma which is beginning to differentiate into muscle. In general, these grafts show the largest amount of normal muscle tissue to be found, although the cases from forty-eight-hour donors tend to be very irregular and incomplete and to contain some degenerating tissue.

Graft 8-18. Right leg bud, including the somites on one side and entire neural cord of the limb level. Seventy-four-hour donor; total age of graft, ten days (fig. 9).

Skeletal parts consist of the limb axis, girdle, and lateral halves of the vertebrae of the limb level. The girdle is formed chiefly of one piece made up of fused ilium, ischium, and pubis, as may be seen in the figure. The head of the femur fits into the opening between the ilium and ischium. The limb cartilages are not normally formed in detail, but femur, tibia-fibular portions, and three digits can be distinguished. Parts are more or less fused.

Muscle development is exceptionally good, although not equal to that which would be found in a ten-day normal embryo. Differentiation is not complete, since striations can be seen only occasionally, but the amount of muscular tissue is much nearer the normal than is common in the grafts. Bundles of fibers extend from the femur to the girdle. Other groups attach even more distally on the limb, extending to what is probably tarsal region, although separate cartilages do not show. One large mass of fibers extends out from the ilium parallel to the femur, but ends in a fascia-like connective tissue with no connection to cartilage. Although rather well-defined tendons appear in the foot region, no connection was seen with any of the muscular elements of the shank. No vacuolated muscular tissue was noted.

The nervous system that is present consists of a segment of the cord with the ganglia and spinal nerves of one side. Four nerve trunks have formed, at least two of which unite in a plexus. All four mingle with the muscle fibers of the limb.

2. Innervation of limb improbable. This group of seven grafts includes those in which it seems very unlikely that the nerve tissue which is present had any appreciable effect upon the limb parts. In three cases a piece of mesencephalon was included with the transplant and in the remaining grafts small groups of spinal-ganglion cells or a fragment of neural tube were noted, but in no case does there appear to be any outgrowth of nerve fibers into the limb region. For this reason these particular grafts will be considered along with those of the following group which do not contain nerves.

B. Nerve tissue absent. 1. Entire limb buds. In this group there are forty grafts including the seven cases mentioned above in section A (2). The donors for these transplants of entire limb rudiments ranged from two days of incubation (two cases) to four and five days (one case each), the great majority being about seventy-two hours old. Most of the grafts reached a total age of from ten to twelve days, although a few were less than that and one was allowed to grow thirteen days before it was preserved.

A rather wide range of development may be seen among the grafts in this group, especially with regard to the skeletal parts. Extremes might be mentioned, one of which may be represented by graft 11-16 (fig. 11) or graft 12-2 (fig. 10), in which the cartilaginous parts are regular and readily recognized. In contrast are the grafts which have small, very irregular masses of cartilage with perhaps recognizable phalanges, but with few other characteristics of a limb. Except for three cases which were rather young and in which all parts were relatively undifferentiated and no muscle had formed, all of the grafts show some muscular development. Not all show normal muscle fibers, however, only groups and masses of vacuolated cells remaining. In even the best cases the muscles have not developed to the extent that they have in the innervated grafts.

Since most of the grafts to be described are figured and show the cartilaginous parts formed, it is unnecessary to describe the skeletal parts in each case. Note will be made of

the muscle development, however. The following five grafts show what remarkably normal skeletons may develop in chorio-allantoic grafts from donors of from two to four days' incubation. The muscle development is fairly typical of the group, although somewhat better than the average. At least six grafts of the group do not show any trace of normal muscle.

Graft 17-23 (fig. 8). The right wing bud and somites of the wing level of a forty-eight-hour donor reached a total age of eleven days. The muscle is not highly differentiated, but a number of fibers are found connecting the humerus with the proximal end of the ulnar cartilage. It is not certain whether all of these fibers are attached at both ends, but some of them are. Groups of degenerated muscle occupy a parallel position and are also scattered about the girdle cartilages. One strand of muscle fibers extends along the radial cartilage.

Graft 6-30 (fig. 6). The left leg bud alone from a donor incubated about sixty-six hours, grown for a total of a little less than ten days. Muscle bundles are found along the femur and connecting across from the large girdle cartilage to the region of the incompletely formed joint between femur and tibia. Other fibers connect this same joint region with the distal end of the tibia. Masses of atypical muscle are found accompanying that which is more normal. It is interesting to note that the girdle cartilage is drawn in toward the femur and that the tibia is bent in a U-shape, much as if they had been modified by the muscular pull exerted upon them. This condition is also found in other grafts. Another point of interest, a fact already noted by Murray ('26), is that bone forms to a much greater thickness on the concave side of a curved cartilage than on the convex side. Rather well-formed tendons occur along the digits, but do not appear to be connected with any of the muscle masses.

Graft 11-16 (fig. 11). The left leg bud with corresponding somites from a seventy-two-hour donor, grown for a total of between ten and eleven days. The muscle found in this graft occurs about the girdle and femur and tibia-fibula and ap-

pears to connect the girdle with other parts of the limb axis. The exact arrangement of muscle strands is not clear, as the mass is heavily infiltrated with red blood corpuscles, but there is some indication of a transition to the vacuolated condition. None of the fibers appear to be very highly differentiated and many look much more pale, swollen, and vesicular than the clear-cut normal fibers which generally show definite fibrillae. Since most of the fibers are cut in cross-section, it is not possible to say whether any dissolution into separate cells has occurred. However, there are some masses present which have definitely lost their fibrous character. The cells contain many vacuoles which are evidently in an early stage of formation, since they are quite small. Although some of the degenerating masses are somewhat isolated in the looser mesenchymal parts of the graft, others appear to be favorably situated for attachment to skeletal parts. Here again tendinous formations are found along the digits, but they do not connect with the muscles.

Graft 4-16 (fig. 7). A right wing bud from a donor slightly older than seventy-two hours was grown for a total of ten days. The muscles of this graft are worthy of note chiefly because they show a more definite grouping into distinct muscle bundles than is generally the case. They are found only along the humerus and are attached at the proximal end to the girdle cartilages. Their mass is considerably below normal. Very little atypical muscle occurs.

Graft 12-2 (fig. 10). A right leg bud from a four-day donor, the graft having grown a total of thirteen days. This implant which produced a well-formed limb skeleton, probably included a considerable amount of somite tissue, since the large basal part of the limb bud was transplanted. However, muscle development is extremely scanty. None occurs around the girdle and only a few fibers are found along the middle of the femur and a few more scattered fibers across between femur and tarsal or tibio-fibular region. Some vacuolated cells are found along the femur and scattered through other parts of the graft, indicating the presence of small

amounts of atypical muscle, but there are no conspicuous masses. It is difficult to account for such a marked absence of muscular tissue in a limb otherwise well formed, unless it might be considered that the graft grew long enough for the muscle to degenerate and almost completely disappear.

2. Partial limb buds. This last group comprises some twenty grafts grown from implants of portions of limb rudiments from donors three to seven days of age. Only one case is from a nine-day embryo and this implant consisted of a mass of thigh muscle transplanted to determine what would happen to fairly well-differentiated tissue when separated from its natural environment. A few groups of much vacuolated cells were all that remained after seven days. The remaining cases show clearly that at least from the third day on, the anlagen of the limb (at least the skeleton) are fairly definitely localized, so that only distal cartilages will form from the distal portion of the bud and the girdle and proximal cartilages from the proximal portion. Not enough material has been accumulated to determine how complete a mosaic the early bud is, but there is some indication that a certain amount of regulation may take place and that a limb bud divided exactly through its proximodistal axis might be expected to yield two essentially complete limb skeletons. Selby and Murray ('28) considered that their experiments showed that the four-day chick limb was a self-differentiating mosaic, although they admit the possibility of the prospective potency exceeding the prospective significance.

Since the total age of these grafts ran as high as fifteen and sixteen days, considerable bone formation is seen and muscle degeneration is rather extensive in practically every case and is invariably present after ten days. Most of the grafts from the basal half of the limb rudiment (which would naturally be expected to have the greatest amount of muscle) developed rather regular girdles and femurs and considerable masses of vacuolated tissue and occasionally a few recognizable muscle fibers.

Certain special features of several grafts may be mentioned. In figure 4, a section of graft 15-28 grown from the posterior half of a right leg bud shows both typical and atypical muscle occurring together, both attached to the diaphysis of the femur and extending along this bone in a parallel direction. The only advantage held by the more normal muscle appears to be its more central position, i.e., closer to the femur. This arrangement is rather generally true, that the normal tissue is closer to the cartilaginous elements with which it is associated and perhaps therefore better situated for attachment. However, the above-mentioned case shows that it is not always possible to explain degeneration by a complete lack of attachment. Figure 5 shows that it is possible for the muscle strands to have an entirely atypical position and still develop normally. A number of muscle fibers, some of which show striations, extend from the femur out to the surface of the graft directly at right angles to the direction that would be expected. These are practically the only recognizable muscle fibers to be found in the graft. It seems somewhat questionable that the attachment afforded by the loose connection with the graft surface would have been comparable to the normal condition. A somewhat similar case is found in graft 24-3, in which some scattered fibers extend out from the girdle cartilage to the surface. They follow such a curved, irregular course that they could scarcely have been maintained under a tension sufficient to be a decisive factor even though one might consider the ends fixed.

DISCUSSION

From these results it may be seen that the skeletal parts of chick limbs grown as chorio-allantoic grafts may attain a remarkably normal differentiation. The mosaic character of the skeletal anlage and its capacity for self-differentiation have already been pointed out by Murray ('26) and Selby and Murray ('28). There is added evidence that the cartilages attain their normal form independent of the muscles as well as other influences—a fact assumed by Murray, although not

supported by his experimental evidence. In our grafts there was sometimes a very normal formation of cartilage and bone in spite of the fact that muscle was practically absent or atypical.

Since muscles frequently develop quite atypically and show a marked tendency to degeneration even in grafts in which the skeleton is relatively normal, it seems reasonable to infer that there is some element of dependence in the development of muscular tissue which is not necessary for the formation of cartilage and bone.

When we look for possible correlative factors, one which immediately presents itself is the innervation. Although there has been considerable controversy as to just how much influence the nervous system has on development in general and particularly on that of skeletal muscle, the evidence from experiments seems to show that early differentiation is independent of nervous stimulation. Harrison ('04) showed that 'nerveless' frog embryos, experimentally produced either by destruction of the central nervous system at an early period or by rearing the embryos in chloretone, developed body musculature that differentiated normally and was capable of functioning. Hooker ('11) obtained similar results. Both observed a certain amount of vacuolization in the fibers, but concluded that this was a sort of oedematous condition due to the general disturbance caused by the operation. Hamburger ('28) reports the formation of a normal limb pattern ('Formbildung') in *Rana* and *Bombinator* embryos following the destruction of the nerve centers which would have innervated the developing limb. All tissues differentiated normally without innervation, but some hypoplasia occurred in the limb as a whole and the muscles were reduced about 50 per cent, probably by atrophy through disuse. No fatty degeneration was observed, although some of a waxy-hyaline type was found. Hamburger was not able to determine whether this degeneration could be attributed directly to a lack of innervation or whether other factors were involved. A marked difference between amphibian and chick muscle with respect to its sen-

sitivity to abnormal influences is suggested by the results of these experiments. Since, as far as the writer is aware, no reference is made in the abundant literature on amphibian limb experiments to regressive changes any more marked than these Hamburger records, it seems that degeneration must occur rarely in this group.

Among the mammals various cases of monster formation in which portions of the central nervous system were lacking and the musculature was correspondingly abnormal may be noted. Perhaps the best known of these are the newborn calf and pig fetuses reported by Weber ('51). In one case the cord and nerves posterior to the thoracic region were lacking, and although hide and hooves were regularly formed, bones were of normal length and thickness, connective tissue, fat, and blood vessels were present; the limb was immovable in all parts due to the fusion of bones, nerves and muscles being entirely absent. The tendons of some of the muscles were dissected out and although they were attached to the usual bones, they ended in fibrous membranes and in place of muscle lamellae there were fat lamellae present. Weber concluded that muscle formation is dependent on the formation of the corresponding nerves, while the development of the other tissues and organs is independent. Herbst ('01) used these observations to support his contention that the nerves exert a definite formative stimulus in the genesis of muscle. Anders ('21) discusses the same material as well as various other cases of human monsters and comes to the opposite conclusion, that originally the muscular tissue had differentiated without any nervous stimulation, but that a secondary degeneration occurred and the muscle fibers were represented by fat lamellae. In view of the work on the Amphibia, this seems the more reasonable explanation.

In the chick very little work has been done on this subject which can be compared to the amphibian experiments or to the observations on mammalian monsters noted above. Murray claims that in chorio-allantoic grafts the nervous system is not an essential factor in the normal development of the

chick limb. In 1926, he stated that "the bud of the three-day chick can complete its development into a limb without any further nervous influence," since he considered that he had successfully excluded all nerve cells in the transplants for his grafts. However, these conclusions were evidently based on observations of the skeletal system alone.

Strangeways and Fell ('26) grew early chick limbs *in vitro* and as subcutaneous grafts in postembryonic chicks and found that although cartilage, bone, and epidermis developed, there was no skeletal muscle present. Since no nerve cells were considered to have been included with the limb rudiment, there should have been no nerves in the limbs grown by tissue-culture methods. The innervation of the grafts would have had to come from the host and the authors state that the chance for such innervation was slight. However, the differentiation attained by these grafted and cultured limb rudiments was so atypical that the absence of nervous tissue can scarcely be considered as a special factor preventing muscle differentiation.

In the present experiments it has been clearly shown that skeletal muscle will differentiate in the absence of nervous tissue. Of thirty-three cases of grafts of entire limb rudiments which do not include nerve cells, twenty-three contain definitely recognizable muscle, some of which is quite normally differentiated and shows striations, although it is generally present in distinctly subnormal amounts. Of the remaining ten, three are too young to be expected to show much differentiation of muscle and all of the others show varying amounts of degenerating muscle. To the grafts already mentioned might be added the seventeen grafts of partial rudiments which were not innervated, but frequently show muscle strands in addition to the degenerated muscle which is so common. It seems reasonable to conclude, then, that in the chick, as in *Amphibia*, skeletal muscle undergoes initial differentiation independent of any nervous influence. This differentiation proceeds at least to the formation of definite fibers showing clearly marked striations and until some de-

gree of division into separate muscle bundles is attained. The absence of a more extensive muscle complex is possibly due to the readiness with which this tissue appears to undergo atrophy and degeneration before any considerable degree of development has been reached.

The only alternate explanation for the differentiation of muscle in the cases cited seems to involve an early contact stimulus of the muscle primordium by nervous tissue before the rudiment was separated from the embryo for transplantation. That is, if the nerves had grown out into the region of the limb anlage before it was removed from the donor, sufficient initial stimulus might have been given to start the differentiation process in the grafted piece. Judging from observation of normal embryos, it seems probable that no direct innervation of the limb bud itself occurs until after the third day. This is particularly true in the case of the posterior limb bud, since in this region the spinal nerves are just beginning to grow out at seventy-two hours. More anteriorly they extend practically to the base of the wing bud, but do not enter it. In spite of the possibility of morphological association, however, it seems scarcely probable that a simple initial contact would be sufficient to start off the entire complicated process of muscle differentiation, especially in consideration of such evidence as Harrison presents to show the evident independence of muscle tissue.

Having presented arguments in support of the fact that the skeletal muscle is independent of the nervous system in its original differentiation, it still remains to determine why this muscle does not continue to develop in a normal fashion. What is the explanation for the subnormal amounts, the absence of definitive muscle groups, and the marked tendency to extreme degeneration? Many of the atypical results might be ascribed to the unfavorable conditions imposed by the operative technique and by growth on the chorio-allantoic membrane. Retardation certainly occurs and growth and differentiation would therefore not be expected to correspond exactly to the normal in point of time. Most of the grafts

developed for a total of ten days or more and yet do not show a muscular development of even an eight-day normal, while the differentiation of feathers and bony tissue is reasonably comparable. Romer ('27, p. 570) states that so far as the thigh is concerned, the musculature of the eight-day chick presents "a miniature replica of the adult", but this observation refers to the grouping of muscle bundles rather than to histological differentiation.

One set of conditions which might well have a considerable influence on a developing graft is that imposed by the blood supply. However, aside from the possibility of hormonal effects or similar blood-borne influences of the host not easily analyzed by the present experiments, it seems probable that the blood supply cannot be held responsible for abnormal muscle. In grafts that are at all successful the supply of vessels appears adequate and not correlated with the occurrence of atypical conditions in the muscle. The masses of degenerated cells, for instance, have a remarkably rich capillary supply and one of the conspicuous things about such tissue is its close association with small blood vessels. A certain amount of stagnation of blood seems to occur rather frequently in grafts and the tissue spaces of localized areas are sometimes filled with red blood corpuscles. That this accumulation of blood cells in an unfavorable condition is indicated by the fact that in extreme cases necrosis may set in and also by the fact that many phagocytes are found about such an area. These cells are evidently actively removing the red blood corpuscles, since their cytoplasm appears filled with fragments of blood cells. Nevertheless, this condition is certainly not specifically associated with muscular tissue, and when it does occur there, the muscle is in many cases relatively normal. The general occurrence of degenerated muscle in grafts of partial rudiments where no stagnation has occurred is further evidence that this is not a primary cause of degeneration.

The question of a possible definite reaction of the host to the graft might be mentioned, although there is no evidence

that such a reaction occurs in limb grafts. That is, no accumulations of leucocytes are found either about the muscles or elsewhere in the grafts except for the phagocytes which are clearly removing red corpuscles. Also the degeneration of the muscle is not a part of a general condition of the graft because other tissues are extremely normal. One case (19-9) showed a general necrosis, perhaps due to the death of the host shortly before the graft was removed from the membrane. The neural-cord tissue that had been included was very necrotic, and even cartilage had undergone regressive changes, while the muscle which was present was intact, showing that skeletal muscle is not especially susceptible to such changes.

A degeneration of muscle occurs as a normal physiological process in the pig embryo (Carey, '22) and in man (Bardeen, '00), although it is not described as a fatty degeneration such as we find in grafts. As far as is known, such a process has not been described in the chick and it was not seen in the normal embryos studied. However, although it is quite reasonable that such changes do take place normally in the formation of the muscle of the adult, it is not to be expected that such a process would account for the extreme results in the grafting experiments.

There appear to remain two possible factors which may be responsible for the maintenance of typical muscle and muscle pattern, first, the relation of muscle to skeletal parts and, secondly, the influence of the nervous system. The former will be discussed first.

In general, the muscle which has remained relatively normal is usually attached at both ends either to the periosteum (or perichondrium) or to fibrous tissue in some way related to skeletal parts or other muscles. The degenerated tissue usually appears to have little or no direct connection with the skeleton, at least at the time of fixation. However, while these statements hold true in general, there are other cases in which the situation is different. Graft 23-18 (fig. 3) may be mentioned as an example in which atypical muscle is at-

tached to cartilage by a tendon. Graft 15-28 (fig. 4) is a similar case where muscle fibers as well as masses of degenerated cells have parallel attachment to the same bone. While such cases appear to show that even attached muscle degenerates, it might be argued that the muscles under consideration were not attached at both ends or had no tensile force acting upon them. It is true that frequently when both normal and degenerated muscle occur alongside of each other, the atypical cells are peripheral to the group of elongated fibers, and it might be suspected that they were also outside the field of influence of postulated stretching factors and therefore have degenerated. Carey ('22) gives such an explanation for the occurrence of a physiological degeneration in the development of muscles in the pig embryo. It would be quite reasonable to suppose that some such condition existed in the grafts from partial rudiments in which muscle primordia were possibly divided between their points of origin and insertion at the time of transplantation. The same might be true for muscles that would normally extend between limb and girdle cartilages when some of these cartilages are missing in the graft. However, this explanation could not be given for the degeneration of muscles for which the normally associated skeletal parts are present and well formed unless it were maintained, as Carey implies, that tension is necessary for muscle development and it could be shown that such tension was lacking.

On consideration of certain examples of apparently normal muscle in the grafts, it is seen that such a condition of tension can scarcely be considered an essential factor. A single graft (24-3) would seem to provide sufficient evidence that it is possible for muscle to differentiate to a considerable extent without mechanical stress and attachment at both ends. In this graft fibers, some of which are striated, are found between a girdle cartilage and the mesenchymal tissue just under the surface of the graft. A few of the fibers may be attached at one end to the cartilage, but at the other end there is nothing but the tissue of the membrane in which the

graft is embedded, which would give an insufficient support in any case. Even if it is considered that these fibers do have a double attachment, there is no indication that there was any tensile force acting, since they curve and bend in entirely different directions in a very inconsistent manner. Nevertheless, as individual fibers they are as well developed as any seen in the grafts and compare very favorably with controls. Similar fibers in grafts 36-9 and 36-11 (fig. 5) might also be mentioned, fibers which are essentially normal but do not have normal relations to skeletal parts and were probably not under an appreciable mechanical stress.

Although it is possible that such mechanical factors of attachment and tensional pull are important in the maintenance of functional muscle, the evidence from the grafts described above seems to the writer to be sufficient to exclude these particular factors as the essential cause of degeneration of muscle in the grafts. It seems likely that attachment to cartilage was much more general before degeneration set in than appears from the grafts, but that when the identity of the fibers was lost, the muscle fascia and tendons were not sufficiently developed in most cases to retain the picture of the attachment. During the degeneration of the muscle fibers as such, the individual cells probably become more embryonic, taking on characteristics of connective-tissue cells in that they store fat and may resemble early stages of adipose tissue. The original aggregations of muscle cells gradually become disorganized, giving rise to the very irregular arrangements in some grafts. As this process continues it is quite likely that the cells are changed into fibrous tissue or else disappear. Evidence of these changes is seen in the grafts and some such process probably accounts for the fact that some grafts have apparently developed so little muscle.

With the elimination of all the other factors which seem to have a possible bearing on the question of muscle degeneration, nervous influence still remains. While the present experiments do not afford entirely conclusive proof, they do indicate that nervous stimulation is important in the main-

tenance of normal musculature after its initial differentiation. This is not a completely unexpected condition. It is well known from observations of clinical and experimental cases that a muscle deprived of its motor nerve supply will atrophy and degenerate unless sufficient artificial stimulation is provided. Electrical excitation, for instance, will keep a muscle in tone until destroyed nerves have regenerated and will again provide a normal stimulus. This appears to be a purely motor effect, the sensory components having no influence. The nature of this muscle-nerve relationship is not very clear, but is usually described as a trophic effect, since without sufficient stimulation the muscle is unable to maintain itself.

In the limb grafts there is evidence of some such nervous effect. Limb rudiments transplanted without nerves have a scanty muscle development which is quite possibly due to an atrophy of the originally differentiated fibers. Such small masses of tissue would be inadequate for splitting into numerous individual muscles, thus accounting for the absence of the typical pattern of muscle groups. In the grafts from older donors, which include chiefly the partial rudiments, some indication of individual muscle masses is seen in the degenerated tissue. In these cases differentiation had proceeded farther before transplantation.

If, in contrast to such grafts, we consider the cases in which nervous tissue is present, we find the situation somewhat different. Of the twenty-eight grafts which are known to contain nerve cells, eleven may be eliminated. Four are too young to be expected to have differentiated muscle and in seven cases any innervation of the limb was so improbable that the grafts were included with those not containing nerve cells. Of the remaining seventeen cases, at least seven are of less successful grafts which were evidently considerably disorganized at implantation. Chiefly axial parts have formed, and whatever muscle is present seems relatively well developed, but has formed principally around the vertebral and girdle cartilages. In all but two cases some degenerated muscle is also present.

The grafts which are the most outstanding of those cited above and having an abundant nerve supply are the remaining ten cases. There seems little doubt that a considerable innervation was supplied to the tissue of all of these grafts. All contained portions of neural cord as well as spinal-ganglion cells and nerve bundles spread throughout the graft. The best-developed graft is 10-10, which has been described in detail above. The muscular tissue is abundant, well differentiated, and divided into distinct bundles with well-developed fibrous tissue associated with them. Nerves can be followed which clearly pass into and become intimately related with the muscle fibers. However, a few small groups of degenerated cells occur even in this graft.

In order to establish the fact that innervation is the factor involved in the degeneration of muscle at least two situations must be explained, first, why degenerated muscle is found in well-innervated grafts and, secondly, why degeneration is not more general in grafts which have no nerves. With respect to the former the suggested interpretation is that the innervation was inadequate either in quantity or quality. It is true that no nerves have been observed which enter a mass of degenerated cells so that it appears that such an explanation has morphological support, but due to the difficulty of tracing nerves in the sections it cannot be maintained arbitrarily that no nerves go to degenerated tissue. Single fibers or even small bundles may possibly have been overlooked. Even in such a case, however, there is still the possibility that such nerves do not have the necessary components, that is, conveying either sensory or motor impulses. No evidence is available from the grafts as to the nature of the nerve supply necessary, but it is to be supposed that certain specific components are concerned, probably the motor elements, as has been mentioned above.

It is more difficult to explain why muscle does not degenerate more completely in non-innervated grafts. A time factor may be involved. The relative amounts of degenerated tissue certainly increase in the older grafts and after ten

days' growth vacuolization of some cells is practically universal. The few cases in which muscle has not degenerated but is atrophied and small in amount can only be interpreted at present as not having reached the stage at which they are completely dependent on some outside influence for maintenance. The fact that they show atrophy and are relatively scarce may indicate a certain degree of dependence even at this stage.

Although the proof is not so clear as can be desired, it seems reasonable to conclude that the muscle of the chick limb, which may undergo an original differentiation without any particular external stimulus, does finally reach a stage at which it can no longer develop independently. If the necessary conditions are not provided, the muscle undergoes regressive changes and will eventually disappear. Although more crucial experiments are required definitely to establish the fact, the evidence from the appearance of muscle tissue in the grafts points to the probability of 'trophic' influences of the nervous system as the most likely factor involved.

Some mention should be made of the development of tendons and such fibrous tissue in the grafts. In the adult fowl the leg muscles are attached to the toes by very long tendons, so that there is scarcely any muscle to be found in the foot region. In the normal embryo these tendons develop simultaneously with the muscles associated with them. In most of the grafts, however, well-defined tendons are lacking, although some exceptions occur, as in graft 10-10 for example, in which the muscle is extensively developed. Tendon-like structures occur in many cases in which digits have formed and usually appear to extend along either or both surfaces of the foot from the end of the foot to the tarsal region, but a normal relationship with muscle is lacking. The evidence seems to suggest that tendons have an independent origin and only connect with their respective muscle bundles later on. However, Levy ('04) states that mechanical pull, as by the gastrocnemius muscle, is essential to the re-formation of the tendon of Achilles in rabbits, and Anders ('21) considers

that the formation of tendons is dependent upon attachment to skeletal muscle whose tonus provided the functional stimulus. It is possible that in the grafts there is actually some connection through the diffuse tissue about the tarsus which establishes a continuity between muscle and tendon, but is not morphologically apparent in sections. This continuity is extremely unlikely, however, and even if a connection did exist, the muscle in some cases would be of the non-functional type. Since an origin of tendons independent of muscular tension is scarcely to be considered without further evidence, this phase of the results must remain inconclusive for the present.

SUMMARY AND CONCLUSIONS

1. The limb rudiments of chick embryos were grown on the chorio-allantoic membrane of older chick hosts. The age of the donor chicks varied from two to seven days of incubation and a series of grafts was obtained in which the total age ranged from six to sixteen days.

2. Tissue for transplantation was isolated in such a way as to include, *a*) the entire limb rudiment alone, *b*) a portion of the rudiment, or, *c*) the limb anlage in combination with, 1) somites of the limb level, 2) somites and nerve cord of the limb level, 3) nerve cord of a different level or, 4) mesencephalon.

3. The muscles of the chick limbs are capable of initial independent differentiation, but they do not invariably maintain this independence. At a certain period, about the tenth day of incubation, some external factor seems necessary to continue the normal development and prevent degeneration.

4. The fatty degeneration which commonly occurs is not considered to be a result of operative technique, a faulty blood supply, an antagonistic reaction of the host, nor a normal physiological process such as is known to occur to a certain extent in some mammals.

5. Since the muscle in the grafts that were not innervated shows fatty degeneration, while in those that were inner-

vated the muscle shows little or no degeneration, it is inferred that innervation is an important factor in the maintenance of normal muscle tissue.

6. Certain mechanical factors such as proper attachment to the normal skeletal structures or a sufficient tension may be involved in the production and maintenance of typical muscle, but some evidence is presented which does not seem to support this hypothesis.

7. The skeleton of the chick limb is essentially self-differentiating, and at least after the third day the anlage appears to be a mosaic.

8. Tendons and other fibrous formations occurred in the grafts to some extent, but frequently did not have a typical relation to muscle and bone.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

3 Photomicrograph of a section from graft 23-18, showing a mass of degenerating muscle (dm) attached by a tendon (t) to cartilage (c). $\times 70$.

4 Photomicrograph of a section from graft 15-28. Both normal muscle (m) and degenerating muscle (dm) occupy a similar position parallel to the femur (f) and appear to be similarly attached. $\times 70$.

5 Photomicrograph of a section of graft 36-11. The normal muscle fibers (m) are attached to the cartilage (c), but extend out to the surface of the graft in a direction perpendicular to that which muscle fibers would be expected to take in that region. Large masses of degenerating muscle (dm) are also seen. $\times 120$.

